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(71) Applicant (*for all designated States except US*): EPIGENOMICS AG [DE/DE]; Kastanienallee 24, 10435 Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): OLEK, Alexander [DE/DE]; Schröderstrasse 13/2, 10115 Berlin (DE). PIEPENBROCK, Christian [DE/DE]; Schwartzkopffstrasse 7b, 10115 Berlin (DE). BERLIN, Kurt [DE/DE]; Marienkäferweg 4, 14532 Stahnsdorf (DE).

(74) Agents: SCHOHE, Stefan et al.; Boehmert & Boehmert, Franz-Joseph-Strasse 38, 80801 München (DE).

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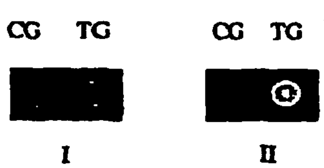
(54) Title: DIAGNOSIS OF DISEASES ASSOCIATED WITH SIGNAL TRANSDUCTION



(57) Abstract: The present invention relates to the chemically modified genomic sequences of genes associated with signal transduction, to oligonucleotides and/or PNA-oligomers for detecting the cytosine methylation state of genes associated with signal transduction which are directed against the sequence, as well as to a method for ascertaining genetic and/or epigenetic parameters of genes associated with signal transduction.

Diagnosis of Diseases Associated with signal transduction

Field of the Invention

The levels of observation that have been well studied by the methodological developments of recent years in molecular biology, are the genes themselves, the translation of these genes into RNA, and the resulting proteins. The question of which gene is switched on at which point in the course of the development of an individual, and how the activation and inhibition of specific genes in specific cells and tissues are controlled is correlatable to the degree and character of the methylation of the genes or of the genome. In this respect, pathogenic conditions may manifest themselves in a changed methylation pattern of individual genes or of the genome.

The present invention relates to nucleic acids, oligonucleotides, PNA-oligomers and to a method for the diagnosis and/or therapy of diseases which have a connection with the genetic and/or epigenetic parameters of genes associated with signal transduction and, in particular, with the methylation status thereof.

Prior Art

Eukaryotic cells exist in a social context, as such, they require a system that allows inter cellular communication. Molecules such as hormones, growth factors and neurotransmitters are utilised as transmitters in cell signalling. These signals allow for the adjustment of factors such as metabolism, growth, proliferation and apoptosis that are essential to the participation of the cell in a social environment. Signal transduction can be defined as the movement of such signals from outside the cell to inside the cell. Signal transmission may be simple, as in the case of acteylcholine receptors which allow the movement of signals through ion channels on the plasma membrane surface. Alternatively, the signals may be transmitted in a more complicated manner through an intracellular signalling cascade by means of protein phosphorylation, (the addition and removal of phosphate groups by protein kinases and protein phosphatases). The system has evolved a high degree of specificity whereby the kinases and phosphatases of the system are extraordinarily precise with respect to their substrates. Signals received at the cell membrane stimulate the activity of a signal integrating complex, which then interacts with substrates, finally

leading to a phenotypic response. Furthermore, evidence is emerging that protein kinases and protein phosphatases work in concert to moderate precise, fast signal transduction that is regulated by feedback.

Signal receptors can be classified into three classes. The first class consists of receptors that span the plasma membrane and which have intrinsic enzymatic activity, such as, tyrosine kinases and guanylate cyclases. The second group consists of, receptors that are coupled to GTP-binding and hydrolyzing proteins, such as odorant receptors and some hormone receptors. The third group comprises intracellular receptors that directly affect gene transcription upon ligand binding.

Disruptions to cell signalling pathways are implicated in many diseases, including cancer, immune disease and inflammatory disorders. Therefore, the elucidation of cell signalling pathways is of considerable importance. The signalling pathways are currently far from completely understood. However, this has not deterred the use of signal transduction as a drug discovery platform, all major pharmaceutical companies currently pursue active drug discovery programs based on signal transduction.

5-methylcytosine is the most frequent covalent base modification in the DNA of eukaryotic cells. It plays a role, for example, in the regulation of the transcription, in genetic imprinting, and in tumorigenesis.

Aberrant DNA methylation within CpG islands is common in human malignancies leading to abrogation or overexpression of a broad spectrum of genes (Jones, P.A. Cancer Res 65:2463-2467, 1996). Abnormal methylation has also been shown to occur in CpG rich regulatory elements in intronic and coding parts of genes for certain tumours (Chan, M.F., et al., Curr Top Microbiol Immunol 249:75-86,2000). Using restriction landmark genomic scanning, Costello and coworkers were able to show that methylation patterns are tumour-type specific (Costello, J. F., et al., Nat Genet 24:132-138, 2000). Highly characteristic DNA methylation patterns could also be shown for breast cancer cell lines (Huang, T. H.-M., et al., Hum Mol Genet 8:459-470, 1999). Genome wide assessment of methylation status represents a molecular fingerprint of cancer tissues.

Therefore, the identification of 5-methylcytosine as a component of genetic information is of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing behavior as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

A relatively new and currently the most frequently used method for analyzing DNA for 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pairing behavior. However, 5-methylcytosine remains unmodified under these conditions. Consequently, the original DNA is converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridization behavior, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridization or sequencing. All of these techniques are based on base pairing which can now be fully exploited. In terms of sensitivity, the prior art is defined by a method which encloses the DNA to be analyzed in an agarose matrix, thus preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and which replaces all precipitation and purification steps with fast dialysis (Olek A, Oswald J, Walter J. A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Res. 1996 Dec 15;24(24):5064-6). Using this method, it is possible to analyze individual cells, which illustrates the potential of the method. However, currently only individual regions of a length of up to approximately 3000 base pairs are analyzed, a global analysis of cells for thousands of possible methylation events is not possible. However, this method cannot reliably analyze very small fragments from small sample quantities either. These are lost through the matrix in spite of the diffusion protection.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein, T., DePamphilis, M. L., Zorbas, H., Nucleic Acids Res. 1998, 26, 2255.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B. A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. Eur J Hum Genet. 1997 Mar-Apr;5(2):94-8)

the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. Nat Genet. 1997 Nov;17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalgo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res, 1997 Jun 15;25(12):2529-31, WO 95/0669) or by enzymatic digestion (Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 1997 Jun 15;25(12):2532-4). In addition, detection by hybridization has also been described (Olek et al., WO 99/8498).

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are: Grigg G, Clark S. Sequencing 5-methylcytosine residues in genomic DNA. Bioessays. 1994 Jun;16(6):431-6, 431; Zeschnigk M, Schmitz B, Dittrich B, Buiting K, Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. Hum Mol Genet. 1997 Mar;6(3):387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res. 1994 Feb 25;22(4):695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R. Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression in human breast cancer cell lines. Gene. 1995 May 19;157(1-2):261-4; WO 97/6705, WO 95/5373 and WO 97/45560.

An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of Nature Genetics (Nature Genetics Supplement, Volume 21, January 1999), published in January 1999, and from the literature cited therein.

Fluorescently labeled probes are often used for the scanning of immobilized DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridized probes may be carried out, for example via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988 Oct 15;60(20):2299-301). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapor phase in an unfragmented manner. The analyte is ionized by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones.

MALDI-TOF spectrometry is excellently suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut I G, Beck S. DNA and Matrix Assisted Laser Desorption Ionization Mass Spectrometry. Current Innovations and Future Trends. 1995, 1; 147-57). The sensitivity to nucleic acids is approximately 100 times worse than to peptides and decreases disproportionally with increasing fragment size. For nucleic acids having a multiply negatively charged backbone, the ionization process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallization. There are now several responsive matrixes for DNA, however, the difference in sensitivity has not been reduced. The difference in sensitivity can be reduced by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. Phosphorothioate nucleic acids in which the usual phosphates of the backbone are substituted with thiophosphates can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut IG, Beck S. A procedure for selective DNA alkylation and detection by mass spectrometry. Nucleic Acids Res. 1995 Apr 25;23(8):1367-73). The coupling of a charge tag to this modified DNA results in an increase in sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities which make the detection of unmodified substrates considerably more difficult.

Genomic DNA is obtained from DNA of cell, tissue or other test samples using standard methods. This standard methodology is found in references such as Fritsch and Maniatis eds., Molecular Cloning: A Laboratory Manual, 1989.

Description

The object of the present invention is to provide the chemically modified DNA of genes associated with signal transduction, as well as oligonucleotides and/or PNA-oligomers for detecting cytosine methylations, as well as a method which is particularly suitable for the diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with signal transduction. The present invention is based on the discovery that genetic and epigenetic parameters and, in particular, the cytosine methylation pattern of genes associated with signal transduction are particularly suitable for the diagnosis and/or therapy of diseases associated with signal transduction.

This objective is achieved according to the present invention using a nucleic acid containing a sequence of at least 18 bases in length of the chemically pretreated DNA of genes associated with signal transduction according to one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto. In the table, after the listed gene designations, the respective data bank numbers (accession numbers) are specified which define the appertaining gene sequences as unique. GenBank was used as the underlying data bank which is located at internet address http://www.ncbi.nlm.nih.gov.

The chemically modified nucleic acid could heretofore not be connected with the ascertainment of genetic and epigenetic parameters.

The object of the present invention is further achieved by an oligonucleotide or oligomer for detecting the cytosine methylation state in chemically pretreated DNA, containing at least one base sequence having a length of at least 13 nucleotides which hybridizes to a chemically pretreated DNA of genes associated with signal transduction according to Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto. The oligomer probes according to the present invention constitute important and effective tools which, for the first time, make it possible to ascertain the genetic and epigenetic parameters of genes associated with signal transduction. The base sequence of the oligomers preferably contains at least one CpG dinucleotide. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Particularly preferred are oligonucleotides according to the present invention in which the cytosine of the CpG dinucleotide is the 5th - 9th nucleotide from

the 5'-end of the 13-mer; in the case of PNA-oligomers, it is preferred for the cytosine of the CpG dinucleotide to be the 4^{th} - 6^{th} nucleotide from the 5'-end of the 9-mer.

The oligomers according to the present invention are normally used in so called "sets" which contain at least one oligomer for each of the CpG dinucleotides of the sequences of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto. Preferred is a set which contains at least one oligomer for each of the CpG dinucleotides from one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto.

Moreover, the present invention makes available a set of at least two oligonucleotides which can be used as so-called "primer oligonucleotides" for amplifying DNA sequences of one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one oligonucleotide is bound to a solid phase.

The present invention moreover relates to a set of at least 10 n (oligonucleotides and/or PNA-oligomers) used for detecting the cytosine methylation state in chemically pretreated genomic DNA (Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto). These probes enable diagnosis and/or therapy of genetic and epigenetic parameters of genes associated with signal transduction. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in the chemically pretreated DNA of genes associated with signal transduction according to one of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto.

According to the present invention, it is preferred that an arrangement of different oligonucleotides and/or PNA-oligomers (a so-called "array") made available by the present invention is pre-

sent in a manner that it is likewise bound to a solid phase. This array of different oligonucleotide- and/or PNA-oligomer sequences can be characterized in that it is arranged on the solid phase in the form of a rectangular or hexagonal lattice. The solid phase surface is preferably composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold. However, nitrocellulose as well as plastics such as nylon which can exist in the form of pellets or also as resin matrices are possible as well.

Therefore, a further subject matter of the present invention is a method for manufacturing an array fixed to a carrier material for analysis in connection with diseases associated with signal transduction in which method at least one oligomer according to the present invention is coupled to a solid phase. Methods for manufacturing such arrays are known, for example, from US Patent 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

A further subject matter of the present invention relates to a DNA chip for the analysis of diseases associated with signal transduction which contains at least one nucleic acid according to the present invention. DNA chips are known, for example, for US Patent 5,837,832.

Moreover, a subject matter of the present invention is a kit which may be composed, for example, of a bisulfite-containing reagent, a set of primer oligonucleotides containing at least two oligonucleotides whose sequences in each case correspond or are complementary to an 18 base long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto), oligonucleotides and/or PNA-oligomers as well as instructions for carrying out and evaluating the described method. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

The present invention also makes available a method for ascertaining genetic and/or epigenetic parameters of genes associated with the cycle cell by analyzing cytosine methylations and single nucleotide polymorphisms, including the following steps:

In the first step of the method, a genomic DNA sample is chemically treated in such a manner that cytosine bases which are unmethylated at the 5'-position are converted to uracil, thymine, or

another base which is dissimilar to cytosine in terms of hybridization behavior. This will be understood as 'chemical pretreatment' hereinafter.

The genomic DNA to be analyzed is preferably obtained form usual sources of DNA such as cells or cell components, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, or combinations thereof.

The above described treatment of genomic DNA is preferably carried out with bisulfite (hydrogen sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to cytosine in terms of base pairing behavior.

Fragments of the chemically pretreated DNA are amplified, using sets of primer oligonucleotides according to the present invention, and a, preferably heat-stable polymerase. Because of statistical and practical considerations, preferably more than ten different fragments having a length of 100 - 2000 base pairs are amplified. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel. Usually, the amplification is carried out by means of a polymerase chain reaction (PCR).

In a preferred embodiment of the method, the set of primer oligonucleotides includes at least two olignonucleotides whose sequences are each reverse complementary or identical to an at least 18 base-pair long segment of the base sequences specified in the appendix (Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto). The primer oligonucleotides are preferably characterized in that they do not contain any CpG dinucleotides.

According to the present invention, it is preferred that at least one primer oligonucleotide is bonded to a solid phase during amplification. The different oligonucleotide and/or PNA-oligomer sequences can be arranged on a plane solid phase in the form of a rectangular or hexagonal lattice, the solid phase surface preferably being composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold, it being possible for other materials such as nitrocellulose or plastics to be used as well.

The fragments obtained by means of the amplification can carry a directly or indirectly detectable label. Preferred are labels in the form of fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer, it being preferred that the fragments that are produced have a single positive or negative net charge for better detectability in the mass spectrometer. The detection may be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The amplificates obtained in the second step of the method are subsequently hybridized to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridization takes place in the manner described in the following. The set of probes used during the hybridization is preferably composed of at least 10 oligonucleotides or PNA-oligomers. In the process, the amplificates serve as probes which hybridize to oligonucleotides previously bonded to a solid phase. The non-hybridized fragments are subsequently removed. Said oligonucleotides contain at least one base sequence having a length of 13 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 5th to 9th nucleotide from the 5'-end of the 13-mer. One oligonucleotide exists for each CpG dinucleotide. Said PNA-oligomers contain at least one base sequence having a length of 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the appendix, the segment containing at least one CpG dinucleotide. The cytosine of the CpG dinucleotide is the 4th to 6th nucleotide seen from the 5'-end of the 9-mer. One oligonucleotide exists for each CpG dinucleotide.

In the fourth step of the method, the non-hybridized amplificates are removed.

In the final step of the method, the hybridized amplificates are detected. In this context, it is preferred that labels attached to the amplificates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

According to the present invention, it is preferred that the labels of the amplificates are fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can

be detected in a mass spectrometer. The mass spectrometer is preferred for the detection of the amplificates, fragments of the amplificates or of probes which are complementary to the amplificates, it being possible for the detection to be carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

The produced fragments may have a single positive or negative net charge for better detectability in the mass spectrometer. The aforementioned method is preferably used for ascertaining genetic and/or epigenetic parameters of genes associated with signal transduction.

The oligomers according to the present invention or arrays thereof as well as a kit according to the present invention are intended to be used for the diagnosis and/or therapy of diseases associated with signal transduction by analyzing methylation patterns of genes associated with signal transduction. According to the present invention, the method is preferably used for the diagnosis and/or therapy of important genetic and/or epigenetic parameters within genes associated with signal transduction.

The method according to the present invention is used, for example, for the diagnosis and/or therapy of solid tumours and cancer. The nucleic acids according to the present invention of Seq. ID No.1 through Seq. ID No.388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to table 1, and sequences complementary thereto can be used for the diagnosis and/or therapy of genetic and/or epigenetic parameters of genes associated with signal transduction.

The present invention moreover relates to a method for manufacturing a diagnostic agent and/or therapeutic agent for the diagnosis and/or therapy of diseases associated with signal transduction by analyzing methylation patterns of genes associated with signal transduction, the diagnostic agent and/or therapeutic agent being characterized in that at least one nucleic acid according to the present invention is used for manufacturing it, possibly together with suitable additives and auxiliary agents.

A further subject matter of the present invention relates to a diagnostic agent and/or therapeutic agent for diseases associated with signal transduction by analyzing methylation patterns of genes

associated with signal transduction, the diagnostic agent and/or therapeutic agent containing at least one nucleic acid according to the present invention, possibly together with suitable additives and auxiliary agents.

The present invention moreover relates to the diagnosis and/or prognosis of events which are disadvantageous to patients or individuals in which important genetic and/or epigenetic parameters within genes associated with signal transduction said parameters obtained by means of the present invention may be compared to another set of genetic and/or epigenetic parameters, the differences serving as the basis for a diagnosis and/or prognosis of events which are disadvantageous to patients or individuals.

In the context of the present invention the term "hybridization" is to be understood as a bond of an oligonucleotide to a completely complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure. To be understood by "stringent hybridization conditions" are those conditions in which a hybridization is carried out at 60°C in 2.5 x SSC buffer, followed by several washing steps at 37°C in a low buffer concentration, and remains stable.

The term "functional variants" denotes all DNA sequences which are complementary to a DNA sequence, and which hybridize to the reference sequence under stringent conditions and have an activity similar to the corresponding polypeptide according to the present invention.

In the context of the present invention, "genetic parameters" are mutations and polymorphisms of genes associated with signal transduction and sequences further required for their regulation. To be designated as mutations are, in particular, insertions, deletions, point mutations, inversions and polymorphisms and, particularly preferred, SNPs (single nucleotide polymorphisms).

In the context of the present invention, "epigenetic parameters" are, in particular, cytosine methylations and further chemical modifications of DNA bases of genes associated with signal transduction and sequences further required for their regulation. Further epigenetic parameters include, for example, the acetylation of histones which, however, cannot be directly analyzed using the described method but which, in turn, correlates with the DNA methylation.

In the following, the present invention will be explained in greater detail on the basis of the sequences and examples wit respect to the accompanying figure without being limited thereto.

Figure 1

Figure 1 shows the hybridisation of fluorescent labelled amplificates to a surface bound oligonucleotide. Sample I being from a astrocytoma tumor sample and sample II being from an oligodenroglioma grade II tumor sample. Flourescence at a spot shows hybridisation of the amplificate to the olignonucleotide. Hybridisation to a CG olignonucleotide denotes methylation at the cytosine position being analysed, hybridisation to a TG olignonucleotide denotes no methylation at the cytosine position being analysed. It can be seen that Sample I had a higher degree of methylation than Sample II.

Seq. ID No. 1 trough Seq. ID No. 388

Sequences having odd sequence numbers (e.g., Seq. ID No. 1, 3, 5, ...) exhibit in each case sequences of the chemically pretreated genomic DNAs of different genes associated with signal transduction. Sequences having even sequence numbers (e.g., Seq. ID No. 2, 4, 6, ...) exhibit in each case the sequences of the chemically pretreated genomic DNAs of genes associated with signal transduction which are complementary to the preceeding sequences (e.g., the complementary sequence to Seq. ID No.1 is Seq. ID No.2, the complementary sequence to Seq. ID No.3 is Seq. ID No.4, etc.)

Seq. ID No. 389 trough Seq. ID No. 392

Seq. ID No. 389 trough Seq. ID No. 392 show sequences of oligonucleotides used in Example 1.

The following example relates to a fragment of a gene associated with signal transduction, in this case, AR in which a specific CG-position is analyzed for its methylation status.

Example 1:Methylation analysis of the gene AR associated with signal transduction.

The following example relates to a fragment of the gene AR in which a specific CG-position is to be analyzed for methylation.

In the first step, a genomic sequence is treated using bisulfite (hydrogen sulfite, disulfite) in such a manner that all cytosines which are not methylated at the 5-position of the base are modified in

such a manner that a different base is substituted with regard to the base pairing behavior while the cytosines methylated at the 5-position remain unchanged.

If bisulfite solution is used for the reaction, then an addition takes place at the non-methylated cytosine bases. Moreover, a denaturating reagent or solvent as well as a radical interceptor must be present. A subsequent alkaline hydrolysis then gives rise to the conversion of non-methylated cytosine nucleobases to uracil. The chemically converted DNA is then used for the detection of methylated cytosines. In the second method step, the treated DNA sample is diluted with water or an aqueous solution. Preferably, the DNA is subsequently desulfonated at an alkaline pH value. In the third step of the method, the DNA sample is amplified in a polymerase chain reaction, preferably using a heat-resistant DNA polymerase. In the present case, cytosines of the gene AR are analyzed. To this end, a defined fragment having a length of 460 bp is amplified with the specific primer oligonucleotides GTAGTAGTAGTAGTAAGAGA (Sequence ID 389) and ACCCCCTAAA TAATTATCCT (Sequence ID No. 390). This amplificate serves as a sample which hybridizes to an oligonucleotide previously bonded to a solid phase, forming a duplex structure, for example TGTTATTTCGAGAGAGGT (Sequence ID No. 391), the cytosine to be detected being located at position 157 of the amplificate. The detection of the hybridization product is based on Cy3 and Cy5 fluorescently labelled primer oligonucleotides which have been used for the amplification. A hybridization reaction of the amplified DNA with the oligonucleotide takes place only if a methylated cytosine was present at this location in the bisulfite-treated DNA. Thus, the methylation status of the specific cytosine to be analyzed is inferred from the hybridization product.

In order to verify the methylation status of the position, a sample of the amplificate is further hybridized to another oligonucleotide previously bonded to a solid phase. Said olignonucleotide is identical to the oligonucleotide previously used to analyze the methylation status of the sample, with the exception of the position in question. At the position to be analysed said oligonucleotide comprises a thymine base as opposed to a cytosine base i.e TGTTATTTTGAGAGAGGT (Sequence ID No. 392). Therefore, the hybridisation reaction only takes place if an unmethylated cytosine was present at the position to be analysed.

Example 2: Diagnosis of diseases associated with signal transduction

In order to relate the methylation patterns to one of the diseases associated with signal transduction, it is initially required to analyze the DNA methylation patterns of a group of diseased and of a group of healthy patients. These analyses are carried out, for example, analogously to Example 1. The results obtained in this manner are stored in a database and the CpG dinucleotides which are methylated differently between the two groups are identified. This can be carried out by determining individual CpG methylation rates as can be done, for example, in a relatively imprecise manner, by sequencing or else, in a very precise manner, by a methylation-sensitive "primer extension reaction". It is also possible for the entire methylation status to be analyzed simultaneously, and for the patterns to be compared, for example, by clustering analyses which can be carried out, for example, by a computer.

Subsequently, it is possible to allocate the examined patients to a specific therapy group and to treat these patients selectively with an individualized therapy.

Example 2 can be carried out, for example, for cancer and solid tumours.

<u>Table 1</u>

List of preferred genes associated with signal transduction according to the invention

Gene	*Genbank Entry No. (http://www.ncbi.nlm.nih.gov)*
DYRK4	Y09305
EPHA5	L36644
NEK3	Z29067
PCTK3	X66362
PRKAR1B	M65066
PRKM3	M84490 Z11696
PRKMK2	L11285
SH3D1B	U61167
ZAP70	L05148
PIK3CA	NM_006218
ADRBK1	NM_001619
AKT1	NM_005163
AKT2	NM_001626
ARHA	NM_001664
BMPR2	NM_001204
CHN1	NM_001822
CHN2	NM_004067
CLK3	NM_003992

Gene	*Genbank Entry No. (http://www.ncbi.nlm.nih.gov)*
CNK	NM_004073
CSK	NM_004383
CSNK1D	NM_001893
CTNNB1	NM_001904
CTNND2	NM_001332
DGKG	NM_001346
DRG2	NM_001388
DVL3	NM_004423
DYRK3	NM_003582
EFNA1	NM_004428
EPHA4	NM_004438
EPHB3	NM_004443
ERBB4	NM_005235
GRB2	NM_002086
HCK	NM_002110
AATK	NM_004920
MADH3	NM_005902
MAPKAPK2	NM_004759
MAP3K3	NM_002401
ROR1	NM_005012
ROR2	NM_004560
PDE4B	NM_002600
PDPK1	NM_002613
B56	NM_006245
PRKACG	NM_002732
PRKAG1	NM_002733
PRKAR1A	NM_002734
PRKCA	NM_002737
PRKCG	NM_002739
PRKCZ	NM_002744
PRKG1	NM_006258
MAPK1	NM_002745
MAPK10	NM_002753
MAPK13	NM_002754
MAPK6	NM_002748
MAPK9	NM_002752
PTK2	NM_005607
PTK2B	NM_004103
RGS7	NM_002924
RHOK	NM_002929
RYK	NM_002958
SFN	NM_006142
STAT1	NM_007315
STATI2	NM_003877
STK3	NM_006281
TIAM1	NM_003253

Gene	*Genbank Entry No. (http://www.ncbi.nlm.nih.gov)*
TTK	NM_003318
TYRO3	NM_006293
UBE1L	NM_003335
YES1	NM_005433
MAP3K12	NM_006301

Claims

1. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated DNA of genes associated with signal transduction according to one of the sequences taken from the group of Seq. ID No.1 to Seq. ID No.388 and sequences complementary thereto.

2. A nucleic acid comprising a sequence at least 18 base pairs in length of a segment of the chemically pretreated DNA of genes associated with signal transduction according to one of the sequences according to the genes CD20 DYRK4 (Y09305),EPHA5 (L36644), NEK3 (Z29067), PCTK3 (X66362), PRKAR1B (M65066), PRKM3 (M84490) (Z11696), PRKMK2 (L11285), SH3D1B (U61167), ZAP70 (L05148), PIK3CA (NM_006218), ADRBK1 (NM_001619), AKT1 (NM_005163), AKT2 (NM_001626), ARHA (NM_001664), BMPR2 (NM_001204), CHN1 (NM_001822),CHN2 (NM_004067), CLK3 (NM_003992), CNK (NM_004073), CSK (NM_004383), CSNK1D (NM_001893), CTNNB1 (NM_001904), CTNND2 (NM_001332), DGKG (NM_001346), DRG2 (NM_001388), DVL3 (NM_004423), DYRK3 (NM_003582), EFNA1 (NM_004428), EPHA4 (NM_004438), EPHB3 (NM_004443), ERBB4 (NM_005235), GRB2 (NM_002086), HCK (NM_002110), AATK (NM_004920), MADH3 (NM_005902),MAPKAPK2 (NM_004759), MAP3K3 (NM_002401), ROR1 (NM_005012), ROR2 (NM_004560), PDE4B (NM_002600), PDPK1 (NM_002613), B56 (NM_006245), PRKACG (NM_002732), PRKAG1 (NM_002733), PRKAR1A (NM_002734), PRKCA (NM_002737), PRKCG (NM_002739), PRKCZ (NM_002744), PRKG1 (NM_006258), MAPK1 (NM_002745), MAPK10 (NM_002753), MAPK13 (NM_002754), MAPK6 (NM_002748), MAPK9 (NM_002752), PTK2 (NM_005607), PTK2B (NM_004103), RGS7 (NM_002924), RHOK (NM_002929), RYK (NM_002958), SFN (NM_006142), STAT1 (NM_007315), STATI2 (NM_003877), STK3 (NM_006281), TIAM1 (NM_003253), TTK (NM_003318), TYRO3 (NM_006293),UBE1L (NM_003335), YES1 (NM_005433), MAP3K12 (NM_006301) and sequences complementary thereto.

3. An oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which hybridizes to or is identical to a chemically pretreated DNA of genes associ-

ated with signal transduction according to one of the Seq ID Nos 1 to 388 according to claim 1 or to a chemically pretreated DNA of genes according to claim 2 and sequences complementary thereto.

4. The oligomer as recited in Claim 3; wherein the base sequence includes at least one CpG dinucleotide.

5. The oligomer as recited in Claim 3; characterized in that the cytosine of the CpG dinucleotide is located approximately in the middle third of the oligomer.

6. A set of oligomers, comprising at least two oligomers according to any of claims 3 to 5.

7. A set of oligomers as recited in Claim 6, comprising oligomers for detecting the methylation state of all CpG dinucleotides within one of the sequences according to Seq. ID Nos. 1 through 388 according to claim 1 or a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto.

8. A set of at least two oligonucleotides as recited in Claim 3, which can be used as primer oligonucleotides for the amplification of DNA sequences of one of Seq. ID 1 through Seq. ID 388 and sequences complementary thereto and/or sequences of a chemically pretreated DNA of genes according to claim 2, and sequences complementary thereto and segments thereof.

9. A set of oligonucleotides as recited in Claim 8, characterized in that at least one oligonucleotide is bound to a solid phase.

10. Use of a set of oligomer probes comprising at least ten of the oligomers according to any of claims 6 through 9 for detecting the cytosine methylation state and/or single nucleotide polymorphisms (SNPs) in a chemically pretreated genomic DNA according to claim 1 or a chemically pretreated DNA of genes according to claim 2.

11. A method for manufacturing an arrangement of different oligomers (array) fixed to a carrier material for analyzing diseases associated with the methylation state of the CpG dinucleo-

tides of one of the Seq. ID 1 through Seq. ID 388 and sequences complementary thereto and/or chemically pretreated DNA of genes according to claim 2, wherein at least one oligomer according to any of the claims 3 through 5 is coupled to a solid phase.

12. An arrangement of different oligomers (array) obtainable according to claim 11.

13. An array of different oligonucleotide- and/or PNA-oligomer sequences as recited in Claim 12, characterized in that these are arranged on a plane solid phase in the form of a rectangular or hexagonal lattice.

14. The array as recited in any of the Claims 12 or 13, characterized in that the solid phase surface is composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold.

15. A DNA- and/or PNA-array for analyzing diseases associated with the methylation state of genes, comprising at least one nucleic acid according to one of the preceeding claims.

16. A method for ascertaining genetic and/or epigenetic parameters for the diagnosis and/or therapy of existing diseases or the predisposition to specific diseases by analyzing cytosine methylations, characterized in that the following steps are carried out:
- in a genomic DNA sample, cytosine bases which are unmethylated at the 5-position are converted, by chemical treatment, to uracil or another base which is dissimilar to cytosine in terms of hybridization behavior;
- fragments of the chemically pretreated genomic DNA are amplified using sets of primer oligonucleotides according to Claim 8 or 9 and a polymerase, the amplificates carrying a detectable label;
- amplificates are hybridized to a set of oligonucleotides and/or PNA probes according to the Claims 6 and 7, or else to an array according to one of the Claims 12 through 15;
the hybridized amplificates are subsequently detected.

17. The method as recited in Claim 16, characterized in that the chemical treatment is carried out by means of a solution of a bisulfite, hydrogen sulfite or disulfite.

18. The method as recited in one of the Claims 16 or 17, characterized in that more than ten different fragments having a length of 100 - 2000 base pairs are amplified.

19. The method as recited in one of the Claims 16 through 18, characterized in that the amplification of several DNA segments is carried out in one reaction vessel.

20. The method as recited in one of the Claims 16 through 19, characterized in that the polymerase is a heat-resistant DNA polymerase.

21. The method as recited in Claim 20, characterized in that the amplification is carried out by means of the polymerase chain reaction (PCR).

22. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are fluorescence labels.

23. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are radionuclides.

24. The method as recited in one of the Claims 16 through 21, characterized in that the labels of the amplificates are detachable molecule fragments having a typical mass which are detected in a mass spectrometer.

25. The method as recited in one of the Claims 16 through 21, characterized in that the amplificates or fragments of the amplificates are detected in the mass spectrometer.

26. The method as recited in one of the Claims 24 and/or 25, characterized in that the produced fragments have a single positive or negative net charge for better detectability in the mass spectrometer

27. The method as recited in one of the Claims 24 through 26, characterized in that detection is carried out and visualized by means of matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

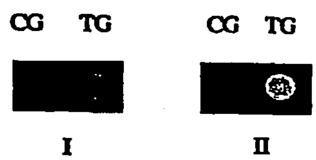
28. The method as recited in one of the Claims 16 through 27, characterized in that the genomic DNA is obtained from cells or cellular components which contain DNA, sources of DNA comprising, for example, cell lines, biopsies, blood, sputum, stool, urine, cerebral-spinal fluid, tissue embedded in paraffin such as tissue from eyes, intestine, kidney, brain, heart, prostate, lung, breast or liver, histologic object slides, and all possible combinations thereof.

29. A kit comprising a bisulfite (= disulfite, hydrogen sulfite) reagent as well as oligonucleotides and/or PNA-oligomers according to one of the Claims 3 through 5.

30. The use of a nucleic acid according to Claims 1 or 2, of an oligonucleotide or PNA-oligomer according to one of the Claims 3 through 5, of a kit according to Claim 29, of an array according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of claims 6 through 9 for the diagnosis of diseases.

31. The use of a nucleic acid according to Claims 1 or 2, of an oligonucleotide or PNA-oligomer according to one of Claims 3 through 5, of a kit according to Claim 29, of an array according to one of the Claims 12 through 15, of a set of oligonucleotides according to one of claims 6 through 9 for the therapy of diseases.

Figur 1



Sequence listing

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HUMANIZED FcγRIIB-SPECIFIC ANTIBODIES AND METHODS OF USE THEREOF

This application claims the benefit of U.S. Provisional Application No. 60/582,043, filed on June 21, 2004, and U.S. Provisional Application No. 60/569,882, filed May 10, 2004, each of which is incorporated herein by reference in its entirety.

1. FIELD OF THE INVENTION

[0001] The present invention relates to humanized FcγRIIB antibodies, fragments, and variants thereof that bind human FcγRIIB with a greater affinity than said antibody binds FcγRIIA. The invention encompasses the use of the humanized antibodies of the invention for the treatment of any disease related to loss of balance of Fc receptor mediated signaling, such as cancer (preferably a B-cell malignancy, particularly, B-cell chronic lymphocytic leukemia or non-Hodgkin's lymphoma), autoimmune disease, inflammatory disease or IgE-mediated allergic disorder. The present invention also encompasses the use of a humanized FcγRIIB antibody or an antigen-binding fragment thereof, in combination with other cancer therapies. The invention provides methods of enhancing the therapeutic effect of therapeutic antibodies by administering the humanized antibodies of the invention to enhance the effector function of the therapeutic antibodies. The invention also provides methods of enhancing the efficacy of a vaccine composition by administering the humanized antibodies of the invention with a vaccine composition.

2. BACKGROUND OF THE INVENTION

2.1 Fc RECEPTORS AND THEIR ROLES IN THE IMMUNE SYSTEM

[0002] The interaction of antibody-antigen complexes with cells of the immune system results in a wide array of responses, ranging from effector functions such as antibody-dependent cytotoxicity, mast cell degranulation, and phagocytosis to immunomodulatory signals such as regulating lymphocyte proliferation and antibody secretion. All these interactions are initiated through the binding of the Fc domain of antibodies or immune complexes to specialized cell surface receptors on hematopoietic cells. The diversity of cellular responses triggered by antibodies and immune complexes results from the structural heterogeneity of Fc receptors. Fc receptors share structurally related ligand binding domains which presumably mediate intracellular signaling.

[0003] The Fc receptors, members of the immunoglobulin gene superfamily of proteins, are surface glycoproteins that can bind the Fc portion of immunoglobulin molecules.

Each member of the family recognizes immunoglobulins of one or more isotypes through a recognition domain on the α chain of the Fc receptor. Fc receptors are defined by their specificity for immunoglobulin subtypes. Fc receptors for IgG are referred to as FcγR, for IgE as FcεR, and for IgA as FcαR. Different accessory cells bear Fc receptors for antibodies of different isotype, and the isotype of the antibody determines which accessory cells will be engaged in a given response (reviewed by Ravetch J.V. *et al.* 1991, *Annu. Rev. Immunol.* 9: 457-92; Gerber J.S. *et al.* 2001 *Microbes and Infection*, 3: 131-139; Billadeau D.D. *et al.* 2002, *The Journal of Clinical Investigation*, 2(109): 161-168; Ravetch J.V. *et al.* 2000, *Science*, 290: 84-89; Ravetch J.V. *et al.*, 2001 *Annu. Rev. Immunol.* 19:275-90; Ravetch J.V. 1994, *Cell*, 78(4): 553-60). The different Fc receptors, the cells that express them, and their isotype specificity is summarized in Table 1 (adapted from Immunobiology: The Immune System in Health and Disease, 4th ed. 1999, Elsevier Science Ltd/Garland Publishing, New York).

Fcγ Receptors

[0004] Each member of this family is an integral membrane glycoprotein, possessing extracellular domains related to a C2-set of immunoglobulin-related domains, a single membrane spanning domain and an intracytoplasmic domain of variable length. There are three known FcγRs, designated FcγRI(CD64), FcγRII(CD32), and FcγRIII(CD16). The three receptors are encoded by distinct genes; however, the extensive homology between the three family members suggest they arose from a common progenitor perhaps by gene duplication. This invention specifically focuses on FcγRII(CD32).

FcγRII(CD32)

[0005] FcγRII proteins are 40KDa integral membrane glycoproteins which bind only the complexed IgG due to a low affinity for monomeric Ig (10^6 M^{-1}). This receptor is the most widely expressed FcγR, present on all hematopoietic cells, including monocytes, macrophages, B cells, NK cells, neutrophils, mast cells, and platelets. FcγRII has only two immunoglobulin-like regions in its immunoglobulin binding chain and hence a much lower affinity for IgG than FcγRI. There are three human FcγRII genes (FcγRII-A, FcγRII-B, FcγRII-C), all of which bind IgG in aggregates or immune complexes.

[0006] Distinct differences within the cytoplasmic domains of FcγRII-A (CD32A) and FcγRII-B (CD32B) create two functionally heterogenous responses to receptor ligation. The fundamental difference is that the A isoform initiates intracellular signaling leading to cell activation such as phagocytosis and respiratory burst, whereas the B isoform initiates inhibitory signals, *e.g.*, inhibiting B-cell activation.

Signaling through FcγRs

[0007] Both activating and inhibitory signals are transduced through the FcγRs following ligation. These diametrically opposing functions result from structural differences among the different receptor isoforms. Two distinct domains within the cytoplasmic signaling domains of the receptor called immunoreceptor tyrosine based activation motifs (ITAMs) or immunoreceptor tyrosine based inhibitory motifs (ITIMS) account for the different responses. The recruitment of different cytoplasmic enzymes to these structures dictates the outcome of the FcγR-mediated cellular responses. ITAM-containing FcγR complexes include FcγRI, FcγRIIA, FcγRIIIA, whereas ITIM-containing complexes only include FcγRIIB.

[0008] Human neutrophils express the FcγRIIA gene. FcγRIIA clustering via immune complexes or specific antibody cross-linking serves to aggregate ITAMs along with receptor-associated kinases which facilitate ITAM phosphorylation. ITAM phosphorylation serves as a docking site for Syk kinase, activation of which results in activation of downstream substrates (*e.g.*, PI_3K). Cellular activation leads to release of proinflammatory mediators.

[0009] The FcγRIIB gene is expressed on B lymphocytes; its extracellular domain is 96% identical to FcγRIIA and binds IgG complexes in an indistinguishable manner. The presence of an ITIM in the cytoplasmic domain of FcγRIIB defines this inhibitory subclass of FcγR. Recently the molecular basis of this inhibition was established. When co-ligated along with an activating FcγR, the ITIM in FcγRIIB becomes phosphorylated and attracts the SH2 domain of the inositol polyphosphate 5'-phosphatase (SHIP), which hydrolyzes phosphoinositol messengers released as a consequence of ITAM-containing FcγR- mediated tyrosine kinase activation, consequently preventing the influx of intracellular Ca^{++}. Thus, crosslinking of FcγRIIB dampens the activating response to FcγR ligation and inhibits cellular responsiveness. B cell activation, B cell proliferation and antibody secretion is thus aborted.

TABLE 1. Receptors for the Fc Regions of Immunoglobulin Isotypes

Receptor	FcγRI (CD64)	FcγRII-A (CD32)	FcγRII-B2 (CD32)	FcγRII-BI (CD32)	FcγRIII (CD16)	FcεRI	FcαRI (CD89)
Binding	IgG1 10^8 M^{-1}	IgG1 2×10^6 M^{-1}	IgG1 2×10^6 M^{-1}	IgG1 2×10^6 M^{-1}	IgG1 5×10^5 M^{-1}	IgG1 10^{10} M^{-1}	IgG1, IgA2 10^7 M^{-1}
Cell Type	Macrophages Neutrophils Eosinophils Dendritic cells	Macrophages Neutrophils Eosinophils Dendritic cells Platelets Langerhan cells	Macrophages Neutrophils Eosinophils	B cells Mast cells	NK cells Eosinophil macrophages Neutrophils Mast Cells	Mast cells Eosinophil Basophils	Macropha ges Neutropils Eosinophi ls
Effect of Ligation	Uptake Stimulation Activation of respiratory burst Induction of killing	Uptake Granule release	Uptake Inhibition of Stimulation	No uptake Inhibition of Stimulation	Induction of Killing	Secretion of granules	Uptake Induction of killing

2.2 DISEASES OF RELEVANCE

2.2.1 CANCER

[0010] A neoplasm, or tumor, is a neoplastic mass resulting from abnormal uncontrolled cell growth which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors are collectively termed cancers. The term "malignant" generally means that the tumor can invade and destroy neighboring body structures and spread to distant sites to cause death (for review, *see* Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-122). Cancer can arise in many sites of the body and behave differently depending upon its origin. Cancerous cells destroy the part of the body in which they originate and then spread to other part(s) of the body where they start new growth and cause more destruction.

[0011] More than 1.2 million Americans develop cancer each year. Cancer is the second leading case of death in the United States and if current trends continue, cancer is expected to be the leading cause of the death by the year 2010. Lung and prostate cancer are the top cancer killers for men in the United States. Lung and breast cancer are the top cancer killers for women in the United States. One in two men in the United States will be diagnosed with cancer at some time during his lifetime. One in three women in the United States will be diagnosed with cancer at some time during her lifetime.

[0012] A cure for cancer has yet to be found. Current treatment options, such as surgery, chemotherapy and radiation treatment, are oftentimes either ineffective or present serious side effects.

2.2.1.1 B-CELL MALIGNANCIES

[0013] B cell malignancies, including, but not limited to, B-cell lymphomas and leukemias, are neoplastic diseases with significant incidence in the United States. There are approximately 55,000 new lymphoma cases of per year in the U.S. (1998 data), with an estimated 25,000 deaths per year. This represents 4% of cancer incidence and 4% of all cancer-related deaths in the U.S. population. The revised European-American classification of lymphoid neoplasms (1994 REAL classification, modified 1999) grouped lymphomas based on their origin as either B cell lineage lymphoma, T cell lineage lymphoma, or Hodgkin's lymphoma. Lymphoma of the B cell lineage is the most common type of non-Hodgkin's lymphoma (NHL) diagnosed in the U.S. (Williams, Hematology 6th ed. (Beutler et al. Ed.), McGraw Hill 2001).

[0014] Chronic lymphocytic leukemia (CLL) is a neoplastic disease characterized by the accumulation of small, mature-appearing lymphocytes in the blood, marrow, and lymphoid

tissues. CLL has an incidence of 2.7 cases per 100,000 in the U.S. The risk increases progressively with age, particularly in men. It accounts for 0.8% of all cancers and is the most common adult leukemia, responsible for 30% of all leukemias. In nearly all cases (>98%) the diseased cells belong to the B lymphocyte lineage. A non-leukemic variant, small lymphocytic lymphoma, constitutes 5-10% of all lymphomas, has histological, morphological and immunological features indistinguishable from that of involved lymph nodes in patients with B-CLL (Williams, 2001).

[0015] The natural history of chronic lymphocytic leukemia falls into several phases. In the early phase, chronic lymphocytic leukemia is an indolent disease, characterized by the accumulation of small, mature, functionally-incompetent malignant B-cells having a lengthened life span. Eventually, the doubling time of the malignant B-cells decreases and patients become increasingly symptomatic. While treatment with chemotherapeutic agents can provide symptomatic relief, the overall survival of the patients is only minimally extended. The late stages of chronic lymphocytic leukemia are characterized by significant anemia and/or thrombocytopenia. At this point, the median survival is less than two years (Foon et al., 1990, Annals Int. Medicine 113:525). Due to the very low rate of cellular proliferation, chronic lymphocytic leukemia is resistant to treatment with chemotherapeutic agents.

[0016] Recently, gene expression studies have identified several genes that may be up regulated in lymphoproliferative disorders. One molecule thought to be over-expressed in patients with B-cell chronic lymphocytic leukemia (B-CLL) and in a large fraction of non-Hodgkin lymphoma patients is CD32B (Alizadeh et al., 2000, Nature 403:503-511; Rosenwald et al., 2001, J. Exp. Med. 184:1639-1647). However, the role of CD32B is B-CLL is unclear since one report demonstrates that CD32B was expressed on a low percentage of B-CLL cells and at a low density (Damle et al., 2002, Blood 99:4087-4093). CD32B is a B cell lineage surface antigen, whose over-expression in B cell neoplasia makes it a suitable target for therapeutic antibodies. In addition, CD32B belongs to the category of inhibitory receptors, whose ligation delivers a negative signal. Therefore, antibodies directed against CD32B could function to eliminate tumor cells by mechanisms that include complement dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), but also triggering an apoptotic signal. The high homology of CD32B with its counterpart, CD32A, an activating Fcã receptor, has thus far hampered the generation of antibodies that selectively recognize one but not the other form of the molecule.

2.2.1.2 <u>Cancer Therapy</u>

[0017] Currently, cancer therapy may involve surgery, chemotherapy, hormonal therapy and/or radiation treatment to eradicate neoplastic cells in a patient (*See*, for example,

Stockdale, 1998, "Principles of Cancer Patient Management", in Scientific American: Medicine, vol. 3, Rubenstein and Federman, eds., Chapter 12, Section IV). Cancer therapy can also involve biological therapy or immunotherapy. All of these approaches pose significant drawbacks for the patient. Surgery, for example, may be contraindicated due to the health of the patient or may be unacceptable to the patient. Additionally, surgery may not completely remove the neoplastic tissue. Radiation therapy is only effective when the neoplastic tissue exhibits a higher sensitivity to radiation than normal tissue, and radiation therapy can also often elicit serious side effects. Hormonal therapy is rarely given as a single agent and, although it can be effective alone, is often used to prevent or delay recurrence of cancer after other treatments have removed the majority of the cancer cells. Biological therapies/immunotherapies are limited in number and may produce side effects such as rashes or swellings, flu-like symptoms, including fever, chills and fatigue, digestive tract problems or allergic reactions.

[0018] With respect to chemotherapy, there are a variety of chemotherapeutic agents available for treatment of cancer. A significant majority of cancer chemotherapeutics act by inhibiting DNA synthesis, either directly, or indirectly by inhibiting the biosynthesis of the deoxyribonucleotide triphosphate precursors, to prevent DNA replication and concomitant cell division (*See*, for example, Gilman *et al.*, Goodman and Gilman's: The Pharmacological Basis of Therapeutics, Eighth Ed. (Pergamom Press, New York, 1990)). These agents, which include alkylating agents, such as nitrosourea, anti-metabolites, such as methotrexate and hydroxyurea, and other agents, such as etoposides, campathecins, bleomycin, doxorubicin, daunorubicin, *etc.*, although not necessarily cell cycle specific, kill cells during S phase because of their effect on DNA replication. Other agents, specifically colchicine and the vinca alkaloids, such as vinblastine and vincristine, interfere with microtubule assembly resulting in mitotic arrest. Chemotherapy protocols generally involve administration of a combination of chemotherapeutic agents to increase the efficacy of treatment.

[0019] Despite the availability of a variety of chemotherapeutic agents, chemotherapy has many drawbacks (*See*, for example, Stockdale, 1998, "Principles Of Cancer Patient Management" in Scientific American Medicine, vol. 3, Rubenstein and Federman, eds., ch. 12, sect. 10). Almost all chemotherapeutic agents are toxic, and chemotherapy causes significant, and often dangerous, side effects, including severe nausea, bone marrow depression, immunosuppression, *etc.* Additionally, even with administration of combinations of chemotherapeutic agents, many tumor cells are resistant or develop resistance to the chemotherapeutic agents. In fact, those cells resistant to the particular chemotherapeutic agents used in the treatment protocol often prove to be resistant to other drugs, even those

agents that act by mechanisms different from the mechanisms of action of the drugs used in the specific treatment; this phenomenon is termed pleiotropic drug or multidrug resistance. Thus, because of drug resistance, many cancers prove refractory to standard chemotherapeutic treatment protocols.

[0020] B cell malignancy is generally treated with single agent chemotherapy, combination chemotherapy and/or radiation therapy. These treatments can reduce morbidity and/or improve survival, albeit they carry significant side effects. The response of B-cell malignancies to various forms of treatment is mixed. For example, in cases in which adequate clinical staging of non-Hodgkin's lymphoma is possible, field radiation therapy can provide satisfactory treatment. Certain patients, however, fail to respond and disease recurrence with resistance to treatment ensues with time, particularly with the most aggressive variants of the disease. About one-half of the patients die from the disease (Devesa et al., 1987, J. Nat'l Cancer Inst. 79:701).

[0021] Investigational therapies for the treatment of refractory B cell neoplasia include autologous and allogeneic bone marrow or stem cell transplantation and gene therapies. Recently, immunotherapy using monoclonal antibodies to target B-cell specific antigens has been introduced in the treatment of B cell neoplasia. The use of monoclonal antibodies to direct radionuclides, toxins, or other therapeutic agents offers the possibility that such agents can be delivered selectively to tumor sites, thus limiting toxicity to normal tissues.

[0022] There is a significant need for alternative cancer treatments, particularly for treatment of cancer that has proved refractory to standard cancer treatments, such as surgery, radiation therapy, chemotherapy, and hormonal therapy. A promising alternative is immunotherapy, in which cancer cells are specifically targeted by cancer antigen-specific antibodies. Major efforts have been directed at harnessing the specificity of the immune response, for example, hybridoma technology has enabled the development of tumor selective monoclonal antibodies (*See* Green M.C. *et al.*, 2000 *Cancer Treat Rev.*, 26: 269-286; Weiner LM, 1999 *Semin Oncol.* 26(suppl. 14):43-51), and in the past few years, the Food and Drug Administration has approved the first MAbs for cancer therapy: Rituxan (anti-CD20) for non-Hodgkin's Lymphoma , Campath (anti-CD52) for B-cell chronic lymphocytic leukemia (B-CLL) and Herceptin [anti-(c-erb-2/HER-2)] for metastatic breast cancer (Suzanne A. Eccles, 2001, Breast Cancer Res. 3: 86-90). NHL and B-CLL are two of the most common forms of B cell neoplasia. These antibodies have demonstrated clinical efficacy, but their use is not without side effects. The potency of antibody effector function, *e.g.*, to mediate antibody dependent cellular cytotoxicity ("ADCC") is an obstacle to such treatment. Furthermore, with

Rituxan and Campath, at least half the patients fail to respond and a fraction of responders may be refractory to subsequent treatments.

[0023] There is a need for alternative therapies for cancer, particularly, B-cell malignancies, especially for patients that are refractory for standard cancer treatments and new immunotherapies such as Rituxan.

2.2.2 INFLAMMATORY DISEASES AND AUTOIMMUNE DISEASES

[0024] Inflammation is a process by which the body's white blood cells and chemicals protect our bodies from infection by foreign substances, such as bacteria and viruses. It is usually characterized by pain, swelling, warmth and redness of the affected area. Chemicals known as cytokines and prostaglandins control this process, and are released in an ordered and self-limiting cascade into the blood or affected tissues. This release of chemicals increases the blood flow to the area of injury or infection, and may result in the redness and warmth. Some of the chemicals cause a leak of fluid into the tissues, resulting in swelling. This protective process may stimulate nerves and cause pain. These changes, when occurring for a limited period in the relevant area, work to the benefit of the body.

[0025] In autoimmune and/or inflammatory disorders, the immune system triggers an inflammatory response when there are no foreign substances to fight and the body's normally protective immune system causes damage to its own tissues by mistakenly attacking itself. There are many different autoimmune disorders which affect the body in different ways. For example, the brain is affected in individuals with multiple sclerosis, the gut is affected in individuals with Crohn's disease, and the synovium, bone and cartilage of various joints are affected in individuals with rheumatoid arthritis. As autoimmune disorders progress, destruction of one or more types of body tissues, abnormal growth of an organ, or changes in organ function may result. The autoimmune disorder may affect only one organ or tissue type or may affect multiple organs and tissues. Organs and tissues commonly affected by autoimmune disorders include red blood cells, blood vessels, connective tissues, endocrine glands (*e.g.*, the thyroid or pancreas), muscles, joints, and skin. Examples of autoimmune disorders include, but are not limited to, Hashimoto's thyroiditis, pernicious anemia, Addison's disease, type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, Sjogren's syndrome, multiple sclerosis, autoimmune inner ear disease, inflammatory bowel disease, arthritis, myasthenia gravis, Reiter's syndrome, Graves disease, autoimmune hepatitis, familial adenomatous polyposis and ulcerative colitis.

[0026] Rheumatoid arthritis (RA) and juvenile rheumatoid arthritis are types of inflammatory arthritis. Arthritis is a general term that describes inflammation in joints. Some,

but not all, types of arthritis are the result of misdirected inflammation. Besides rheumatoid arthritis, other types of arthritis associated with inflammation include the following: psoriatic arthritis, Reiter's syndrome, ankylosing spondylitis arthritis, and gouty arthritis. Rheumatoid arthritis is a type of chronic arthritis that occurs in joints on both sides of the body (such as both hands, wrists or knees). This symmetry helps distinguish rheumatoid arthritis from other types of arthritis. In addition to affecting the joints, rheumatoid arthritis may occasionally affect the skin, eyes, lungs, heart, blood or nerves.

[0027] Rheumatoid arthritis affects about 1% of the world's population and is potentially disabling. There are approximately 2.9 million incidences of rheumatoid arthritis in the United States. Two to three times more women are affected than men. The typical age that rheumatoid arthritis occurs is between 25 and 50. Juvenile rheumatoid arthritis affects 71,000 young Americans (aged eighteen and under), affecting six times as many girls as boys.

[0028] Rheumatoid arthritis is an autoimmune disorder where the body's immune system improperly identifies the synovial membranes that secrete the lubricating fluid in the joints as foreign. Inflammation results, and the cartilage and tissues in and around the joints are damaged or destroyed. In severe cases, this inflammation extends to other joint tissues and surrounding cartilage, where it may erode or destroy bone and cartilage and lead to joint deformities. The body replaces damaged tissue with scar tissue, causing the normal spaces within the joints to become narrow and the bones to fuse together. Rheumatoid arthritis creates stiffness, swelling, fatigue, anemia, weight loss, fever, and often, crippling pain. Some common symptoms of rheumatoid arthritis include joint stiffness upon awakening that lasts an hour or longer; swelling in a specific finger or wrist joints; swelling in the soft tissue around the joints; and swelling on both sides of the joint. Swelling can occur with or without pain, and can worsen progressively or remain the same for years before progressing.

[0029] The diagnosis of rheumatoid arthritis is based on a combination of factors, including: the specific location and symmetry of painful joints, the presence of joint stiffness in the morning, the presence of bumps and nodules under the skin (rheumatoid nodules), results of X-ray tests that suggest rheumatoid arthritis, and/or positive results of a blood test called the rheumatoid factor. Many, but not all, people with rheumatoid arthritis have the rheumatoid-factor antibody in their blood. The rheumatoid factor may be present in people who do not have rheumatoid arthritis. Other diseases can also cause the rheumatoid factor to be produced in the blood. That is why the diagnosis of rheumatoid arthritis is based on a combination of several factors and not just the presence of the rheumatoid factor in the blood.

[0030] The typical course of the disease is one of persistent but fluctuating joint symptoms, and after about 10 years, 90% of sufferers will show structural damage to bone and

cartilage. A small percentage will have a short illness that clears up completely, and another small percentage will have very severe disease with many joint deformities, and occasionally other manifestations of the disease. The inflammatory process causes erosion or destruction of bone and cartilage in the joints. In rheumatoid arthritis, there is an autoimmune cycle of persistent antigen presentation, T-cell stimulation, cytokine secretion, synovial cell activation, and joint destruction. The disease has a major impact on both the individual and society, causing significant pain, impaired function and disability, as well as costing millions of dollars in healthcare expenses and lost wages (*see,* for example, the NIH website and the NIAID website).

[0031] Currently available therapy for arthritis focuses on reducing inflammation of the joints with anti-inflammatory or immunosuppressive medications. The first line of treatment of any arthritis is usually anti-inflammatories, such as aspirin, ibuprofen and Cox-2 inhibitors such as celecoxib and rofecoxib. "Second line drugs" include gold, methotrexate and steroids. Although these are well-established treatments for arthritis, very few patients remit on these lines of treatment alone. Recent advances in the understanding of the pathogenesis of rheumatoid arthritis have led to the use of methotrexate in combination with antibodies to cytokines or recombinant soluble receptors. For example, recombinant soluble receptors and monoclonal antibodies for tumor necrosis factor (TNF)-α have been used in combination with methotrexate in the treatment of arthritis. However, only about 50% of the patients treated with a combination of methotrexate and anti-TNF-α agents such as recombinant soluble receptors for TNF-α show clinically significant improvement. Many patients remain refractory despite treatment. Difficult treatment issues still remain for patients with rheumatoid arthritis. Many current treatments have a high incidence of side effects or cannot completely prevent disease progression. So far, no treatment is ideal, and there is no cure. Novel therapeutics are needed that more effectively treat rheumatoid arthritis and other autoimmune disorders.

2.2.3 ALLERGY

[0032] Immune-mediated allergic (hypersensitivity) reactions are classified into four types (I-IV) according to the underlying mechanisms leading to the expression of the allergic symptoms. Type I allergic reactions are characterized by IgE-mediated release of vasoactive substances such as histamine from mast cells and basophils. The release of these substances and the subsequent manifestation of allergic symptoms are initiated by the cross-linking of allergen-bound IgE to its receptor on the surface of mast cells and basophils. In individuals suffering from type I allergic reactions, exposure to an allergen for a second time leads to the production of high levels of IgE antibodies specific for the allergen as a result of the involvement of memory B and T cells in the 3-cell interaction required for IgE production.

The high levels of IgE antibodies produced cause an increase in the cross-linking of IgE receptors on mast cells and basophils by allergen-bound IgE, which in turn leads to the activation of these cells and the release of the pharmacological mediators that are responsible for the clinical manifestations of type I allergic diseases.

[0033] Two receptors with differing affinities for IgE have been identified and characterized. The high affinity receptor (FcεRI) is expressed on the surface of mast cells and basophils. The low affinity receptor (FcεRII/CD23) is expressed on many cell types including B cells, T cells, macrophages, eosinophils and Langerhan cells. The high affinity IgE receptor consists of three subunits (alpha, beta and gamma chains). Several studies demonstrate that only the alpha chain is involved in the binding of IgE, whereas the beta and gamma chains (which are either transmembrane or cytoplasmic proteins) are required for signal transduction events. The identification of IgE structures required for IgE to bind to the FcεRI on mast cells and basophils is of utmost importance in devising strategies for treatment or prevention of IgE-mediated allergies. For example, the elucidation of the IgE receptor-binding site could lead to the identification of peptides or small molecules that block the binding of IgE to receptor-bearing cells *in vivo*.

[0034] Currently, IgE-mediated allergic reactions are treated with drugs such as antihistamines and corticosteroids which attempt to alleviate the symptoms associated with allergic reactions by counteracting the effects of the vasoactive substances released from mast cells and basophils. High doses of antihistamines and corticosteroids have deleterious side effects (*e.g.*, central nervous system disturbance, constipation, *etc*). Thus, other methods for treating type I allergic reactions are needed.

[0035] One approach to the treatment of type I allergic disorders has been the production of monoclonal antibodies which react with soluble (free) IgE in serum, block IgE from binding to its receptor on mast cells and basophils, and do not bind to receptor-bound IgE (*i.e.*, they are non-anaphylactogenic). One such antibody Xolair, has been approved by the FDA.

[0036] One of the most promising treatments for IgE-mediated allergic reactions is the active immunization against appropriate non-anaphylactogenic epitopes on endogenous IgE. Stanworth *et al.* (U.S. Patent No. 5,601,821) described a strategy involving the use of a peptide derived from the CεH4 domain of the human IgE coupled to a heterologous carrier protein as an allergy vaccine. However, this peptide has been shown not to induce the production of antibodies that react with native soluble IgE. Further, Hellman (U.S. Patent No. 5,653,980) proposed anti-IgE vaccine compositions based on fusion of full length CεH2-CεH3 domains (approximately 220 amino acid long) to a foreign carrier protein. However, the antibodies

induced by the anti-IgE vaccine compositions proposed in Hellman will most likely it result in anaphylaxis since antibodies against some portions of the CεH2 and CεH3 domains of the IgE molecule have been shown to cross-link the IgE receptor on the surface of mast cell and basophils and lead to production of mediators of anaphylaxis (*See*, *e.g.*, Stadler *et al.*, 1993, *Int. Arch. Allergy and Immunology* 102:121-126). Therefore, a need remains for treatment of IgE-mediated allergic reactions which do not induce anaphylactic antibodies.

[0037] The significant concern over induction of anaphylaxis has resulted in the development of another approach to the treatment of type I allergic disorders consisting of mimotopes that could induce the production of anti-IgE polyclonal antibodies when administered to animals (*See, e.g.*, Rudolf, *et al.*, 1998, Journal of Immunology 160:3315-3321). Kricek *et al.* (International Publication No. WO 97/31948) screened phage-displayed peptide libraries with the monoclonal antibody BSWI7 to identify peptide mimotopes that could mimic the conformation of the IgE receptor binding. These mimotopes could presumably be used to induce polyclonal antibodies that react with free native IgE, but not with receptor-bound IgE as well as block IgE from binding to its receptor. Kriek *et al.* disclosed peptide mimotopes that are not homologous to any part of the IgE molecule and are thus different from peptides disclosed in the present invention.

[0038] As evidenced by a survey of the art, there remains a need for enhancing the therapeutic efficacy of current methods of treating or preventing disorders such as cancer, autoimmune disease, inflammatory disorder, or allergy. In particular, there is a need for enhancing the effector function, particularly, the cytotoxic effect of therapeutic antibodies used in treatment of cancer. The current state of the art is also lacking in treating or preventing allergy disorders (*e.g.*, either by antibody therapy or vaccine therapy).

3. <u>SUMMARY OF THE INVENTION</u>

[0039] The instant invention provides humanized FcγRIIB antibodies, an isolated antibody or a fragment thereof that specifically binds FcγRIIB, particularly human FcγRIIB, more particularly native human FcγRIIB, with a greater affinity than said antibody or a fragment thereof binds FcγRIIA, particularly human FcγRIIA, more particularly native human FcγRIIA. As used herein, "native FcγRIIB or FcγRIIA " means FcγRIIB or FcγRIIA which is endogenously expressed in a cell and is present on the cell surface of that cell or recombinantly expressed in a mammalian cell and present on the cell surface, but is not FcγRIIB or FcγRIIA expressed in a bacterial cell or denatured, isolated FcγRIIB or FcγRIIA. The instant invention encompasses humanized antibodies, and antigen binding fragments thereof, derived from antibodies that bind FcγRIIB, particularly human FcγRIIB, more particularly native human FcγRIIB, with a greater affinity than said antibody or a fragment thereof binds FcγRIIA,

particularly human FcγRIIA, more particularly native human FcγRIIA. In most preferred embodiments, the instant invention relates to humanized 2B6 or 3H7 antibodies or fragments thereof, preferably antigen binding fragments thereof. In another preferred embodiments, the invention relates to humanized 1D5, 2E1, 2H9, 2D11, or 1F2 antibodies and fragments thereof, preferably antigen binding fragments thereof.

[0040] Preferably the humanized antibodies of the invention bind the extracellular domain of native human FcγRIIB. The humanized anti- FcγRIIB antibodies of the invention may have a heavy chain variable region comprising the amino acid sequence of CDR1 (SEQ ID NO. 1 or SEQ ID NO. 29) and/or CDR2 (SEQ ID NO. 2 or SEQ ID NO.30) and/or CDR3 (SEQ ID NO. 3 or SEQ ID NO. 31) and/or a light chain variable region comprising the amino acid sequence of CDR1 (SEQ ID NO. 8 or SEQ ID NO. 38) and/or a CDR2 (SEQ ID NO. 9, SEQ ID NO. 10, SEQ ID NO. 11, or SEQ ID NO. 39) and/or CDR3 (SEQ ID NO. 12 or SEQ ID NO. 40).

[0041] In yet other preferred embodiments, the humanized antibodies of the invention comprise a light chain variable regions comprising an amino acid sequence of SEQ ID NO. 18, SEQ ID NO. 20, SEQ ID NO. 22, or SEQ ID NO. 46, and/or a heavy chain variable region comprising the amino acid sequence of SEQ ID NO. 24 or SEQ ID NO. 37, and/or amino acid sequence variants thereof.

[0042] In particular, the invention provides a humanized antibody that immunospecifically binds to extracellular domain of native human FcγRIIB, said antibody comprising (or alternatively, consisting of) a VH CDR1 and a VL CDR1; a VH CDR1 and a VL CDR2; a VH CDR1 and a VL CDR3; a VH CDR2 and a VL CDR1; VH CDR2 and VL CDR2; a VH CDR2 and a VL CDR3; a VH CDR3 and a VH CDR1; a VH CDR3 and a VL CDR2; a VH CDR3 and a VL CDR3; a VH1 CDR1, a VH CDR2 and a VL CDR1; a VH CDR1, a VH CDR2 and a VL CDR2; a VH CDR1, a VH CDR2 and a VL CDR3; a VH CDR2, a VH CDR3 and a VL CDR1, a VH CDR2, a VH CDR3 and a VL CDR2; a VH CDR2, a VH CDR2 and a VL CDR3; a VH CDR1, a VL CDR1 and a VL CDR2; a VH CDR1, a VL CDR1 and a VL CDR3; a VH CDR2, a VL CDR1 and a VL CDR2; a VH CDR2, a VL CDR1 and a VL CDR3; a VH CDR3, a VL CDR1 and a VL CDR2; a VH CDR3, a VL CDR1 and a VL CDR3; a VH CDR1, a VH CDR2, a VH CDR3 and a VL CDR1; a VH CDR1, a VH CDR2, a VH CDR3 and a VL CDR2; a VH CDR1, a VH CDR2, a VH CDR3 and a VL CDR3; a VH CDR1, a VH CDR2, a VL CDR1 and a VL CDR2; a VH CDR1, a VH CDR2, a VL CDR1 and a VL CDR3; a VH CDR1, a VH CDR3, a VL CDR1 and a VL CDR2; a VH CDR1, a VH CDR3, a VL CDR1 and a VL CDR3; a VH CDR2, a VH CDR3, a VL CDR1 and a VL CDR2; a VH CDR2, a VH CDR3, a VL CDR1 and a VL CDR3; a VH CDR2, a VH CDR3, a VL

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[0043] In one specific embodiment, the invention provides a humanized 2B6 antibody, wherein the VH region consists of the FR segments from the human germline VH segment VH1-18 and JH6, and the CDR regions of the 2B6 VH, having the amino acid sequence of SED ID NO. 24. In another specific embodiment, the humanized 2B6 antibody further comprises a VL regions, which consists of the FR segments of the human germline VL segment VK-A26 and JK4 and the CDR regions of 2B6VL, having an amino acid sequence of SEQ ID NO. 18, SEQ ID NO. 20, or SEQ ID NO. 22.

[0044] In one specific embodiment, the invention provides a humanized 3H7 antibody, wherein the VH region consists of the FR segments from a human germline VH segment, and the CDR regions of the 3H7 VH, having the amino acid sequence of SED ID NO. 37. In another specific embodiment, the humanized 3H7 antibody further comprises a VL regions, which consists of the FR segments of the human germline VL segment, and the CDR regions of 3H7VL, having an amino acid sequence of SEQ ID NO. 46.

[0045] The present invention provides humanized antibody molecules specific for FcγRIIB in which one or more regions of one or more CDRs of the heavy and/or light chain variable regions of a human antibody (the recipient antibody) have been substituted by analogous parts of one or more CDRs of a donor monoclonal antibody which specifically binds FcγRIIB, with a greater affinity than FcγRIIA, *e.g.*, monoclonal antibody produced by clones 2B6 and 3H7 which bind FcγRIIB, having ATCC accession numbers PTA-4591, and PTA-4592, respectively, or a monoclonal antibody produced by clones 1D5, 2E1, 2H9, 2D11, and 1F2 having ATCC Accession numbers, PTA-5958, PTA-5961, PTA-5962, PTA-5960, and PTA-5959, respectively. In a most preferred embodiment, the humanized antibody can specifically bind to the same epitope as the donor murine antibody. It will be appreciated by one skilled in the art that the invention encompasses CDR grafting of antibodies in general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

[0046] In some embodiments, at least one CDR from the donor antibody is grafted onto the human antibody. In other embodiments, at least two and preferably all three CDRs of each

of the heavy and/or light chain variable regions are grafted onto the human antibody. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a combination thereof. In some embodiments, the invention encompasses a humanized FcγRIIB antibody comprising at least one CDR grafted heavy chain and at least one CDR-grafted light chain.

[0047] In a preferred embodiment, the CDR regions of the humanized FcγRIIB specific antibody are derived from the murine antibody against FcγRIIB. In some embodiments, the humanized antibodies described herein comprise alterations, including, but not limited to, amino acid deletions, insertions, and modifications, of the acceptor antibody, *i.e.*, human, heavy and/or light chain variable domain framework regions that are necessary for retaining binding specificity of the donor monoclonal antibody. In some embodiments, the framework regions of the humanized antibodies described herein do not necessarily consist of the precise amino acid sequence of the framework region of a natural occurring human antibody variable region, but contain various alterations, including, but not limited to, amino acid deletions, insertions, modifications that alter the property of the humanized antibody, for example,

SEQ ID 74 from WO/02 00926

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